The *Trp53-Trp53inp1-Tnfrsf10b* Pathway Regulates the Radiation Response of Mouse Spermatogonial Stem Cells

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SUMMARY

Germ cells are thought to exhibit a unique DNA damage response that differs from that of somatic stem cells, and previous studies suggested that *Trp53* is not involved in the survival of spermatogonial stem cells (SSCs) after irradiation. Here, we report a critical role for the *Trp53-Trp53inp1-Tnfrsf10b* pathway during radiation-induced SSC apoptosis. Spermatogonial transplantation revealed that *Trp53* deficiency increased the survival of SSCs after irradiation. Although *Bbc3*, a member of the intrinsic apoptotic pathway, was implicated in apoptosis of germ and somatic stem cells, *Bbc3* depletion inhibited apoptosis in committed spermatogonia, but not in SSCs. In contrast, inhibition of *Tnfrsf10b*, an extrinsic apoptosis regulator, rescued SSCs. *Tnfrsf10b*, whose deficiency protected SSCs, was upregulated by *Trp53inp1* upon irradiation. These results suggest that the *Trp53-Trp53inp1-Tnfrsf10b* pathway responds to genotoxic damage in SSCs and that stem and progenitor cells exhibit distinct DNA damage responses in self-renewing tissue.

INTRODUCTION

The testis is a tissue that is highly sensitive to DNA damage by ionizing radiation. Compared with somatic cells in the testis, spermatogenic cells are easily damaged by radiation insults: irradiated animals undergo germ cell loss and become sterile (Meistrich, 1982; Creemers et al., 2002). However, depending on the radiation dose, spermatogenesis can regenerate to regain fertility (Meistrich et al., 1978). Spermatogonial stem cells (SSCs) are important for regeneration. Although there are only $2-3 \times 10^4$ SSCs in the testis (Meistrich and van Beek, 1993; de Rooij and Russell, 2000), their robust regenerative activity supports spermatogenesis throughout adult life.

It is generally believed that germ cells have a lower mutation rate than somatic cells (Provost et al., 1993; Walter et al., 1998; Hill et al., 2004). Moreover, the survival of spermatogonia after radiation damage varies depending on their stage of differentiation. Differentiating spermatogonia (including A_1-A_4, intermediate, and B spermatogonia) are the most sensitive, whereas undifferentiated spermatogonia (including SSCs) can survive moderate radiation doses (Erickson, 1976; Dym and Clermont, 1970). A relatively higher apoptosis rate of progenitors has also been reported in other self-renewing tissues (Etienne et al., 2012; Qiu et al., 2008), but the mechanism for this remains unclear.

Double-strand breaks (DSBs), which are created by radiation and are the most hazardous type of DNA damage, are generally repaired by nonhomologous end joining (NHEJ) and homologous recombination (HR) (Branzei and Foiani, 2008). Whereas NHEJ is error prone and functions throughout the cell cycle, HR is error free and occurs in S and G2 phases when sister chromatids are available as templates. The cellular response is initiated by ataxia telangiectasia-mutated (ATM) and DNA protein kinase, which associates with DSBs and phosphorylates histone H2AX. Phosphorylated H2AX (γH2AX) recruits damage repair proteins such as MDC1. Additional factors, such as 53BP1, then bind and initiate DNA repair (Eliezer et al., 2009).

However, several studies have suggested that a unique DNA repair mechanism operates in SSCs. γH2AX is not detected in undifferentiated spermatogonia, possibly including SSCs, whereas differentiated spermatogonia exhibit distinct foci formation (Rübe et al., 2011). It was shown that these cells also do not express MDC1 after irradiation, although nuclear 53BP1 foci were detected (Ahmed et al., 2007; Rübe et al., 2011). More surprisingly, several groups suggested that the tumor suppressor *Trp53*, a key molecule in the response to DNA damage, does not play a role in the radiation-induced apoptosis of SSCs (Hendry et al., 1996; Beumer et al., 1998; Hasegawa et al., 1998). In *Trp53* knockout (KO) mice, negligible spermatogonia apoptosis was observed after doses of up to 5 Gy, whereas the number of spermatogonia was reduced by 60% within 1 day in wild-type (WT) mice (Beumer et al., 1998). In addition, TRP53 was not detected in undifferentiated spermatogonia in either nonirradiated or irradiated conditions. Therefore, the reduction in the number of spermatogonia resulted from *Trp53*-dependent apoptosis

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in differentiating spermatogonia (Beumer et al., 1998; Hasegawa et al., 1998).

Traditionally, the effects of radiation on spermatogonia have been evaluated by morphological analyses. It is considered that the percentage of seminiferous tubule cross-sections that show type A spermatogonia 10 days after irradiation is representative of SSC survival (van der Meer et al., 1992). Among the several different types of undifferentiated spermatogonia, A_{single} (A_s) spermatogonia are thought to have SSC potential (de Rooij and Russell, 2000). However, it is difficult to distinguish SSCs from committed progenitors morphologically. This is particularly true after genotoxic stress, which may influence the cell cycle and morphology. Moreover, because germ cells contact Sertoli cells directly within seminiferous tubules, the potential effects of paracrine interactions with neighboring peritubular and interstitial cells must be considered. Radiation-induced damage to Sertoli cells causes hormonal imbalance and dysregulated cytokine secretion (Guitton et al., 1999; Legué et al., 2001), which impairs the analysis of SSCs after irradiation.

In this study, we used two techniques to investigate the mechanism of radiation-induced apoptosis of SSCs. First, we used germ cell transplantation. Transplantation of testis cells into the seminiferous tubules of infertile testes results in the regeneration of spermatogenesis from donor SSCs (Brinster and Zimmermann, 1994). This allows the functional identification of SSCs. Second, we used an SSC culture technique (Kanatsu-Shinohara et al., 2003). SSCs undergo self-renewal division and proliferate as grape-like clusters of spermatogonia when the media are supplemented with fibroblast growth factor 2 (FGF2) and glial cell line-derived neurotrophic factor (GDNF). These cultured spermatogonia, designated germline stem cells (GSCs), are enriched for SSCs. Assuming 10% colonization efficiency (Nagano et al., 1999), 1%–2% of GSCs can colonize seminiferous tubules (Kanatsu-Shinohara et al., 2005). GSCs proliferate logarithmically and recolonize seminiferous tubules, resulting in spermatogenesis and normal offspring. Because it is possible to manipulate GSCs genetically, the combination of transplantation and culture techniques for SSCs provides a powerful approach for investigating the effect of candidate genes involved in radiation response. Using these strategies, we assessed the molecular mechanism underlying the DNA damage response in SSCs.

RESULTS

Increased SSC Survival in *Trp53* KO Mice
To examine the impact of radiation on SSCs, we irradiated the testes of transgenic mice (C57BL6/Tg14(act-EGFP-OsbY01), termed "green mice") that expressed enhanced

GFP (EGFP). Green mice with the WT and *Trp53* KO genotypes were used for transplantation. The testes of recipient animals were recovered 2 months after transplantation and the number of donor-cell-derived colonies was counted under UV light. More colonies were derived from *Trp53* KO testis cells at all tested radiation doses than from WT cells. The difference was statistically significant when cells were irradiated at >4 Gy (Figures 1A and 1B). We also evaluated the effect of cell dissociation. Dissociated testis cells at this dose had a higher apoptotic rate than intact testes that were irradiated before dissociation (Figure 1C), suggesting that the seminiferous tubule structure confers some SSC radioprotection.

We next used GSCs to compare their radiation response with that of mouse embryonic fibroblasts (MEFs) and multipotent GSCs (mGSCs), which are derived from GSCs and exhibit embryonic stem cell (ESC)-like properties (Kanatsu-Shinohara et al., 2004; Figure 1D). Cells were recovered at different time points to determine the time course of cell death. GSCs exhibited the highest sensitivity to irradiation: survival decreased in a dose-dependent manner and the rate of cell recovery was delayed significantly compared with other cell types. In contrast, mGSCs and MEFs showed a transient decrease after irradiation, but the cell number then increased by 3 days postirradiation. Cell recovery was minimal at 72, 12, and 24 hr for GSCs, mGSCs, and MEFs, respectively. We then used these time points to determine the lethal dose (LD_{50}) value for each cell type (Figure 1E). As expected from the transplantation experiments, apoptosis was attenuated significantly by *Trp53* deficiency. The LD_{50} for WT and *Trp53* KO GSCs was 1.5 and 3.6 Gy, respectively. The survival rate of GSCs was comparable in at least two genetic backgrounds (Figure S1A available online). The LD_{50} values for mGSCs and ESCs were also comparable (3.8 versus 4.2 Gy, respectively; Figure S1B). MEFs were the least sensitive, with an LD_{50} of 6.2 Gy. These LD_{50} values were used in subsequent experiments.

We confirmed the effect of radiation on GSCs using transplantation. GSCs from WT and *Trp53* KO mice were labeled with a lentivirus expressing *Venus* to introduce a donor cell marker. GSCs were irradiated at 1.5 and 3.6 Gy. Significantly more *Trp53* KO GSCs survived compared with WT GSCs (Figures 1F and 1G). Western blotting revealed that irradiation increased the expression of TRP53 and its phosphorylation at Ser18 and Ser23, which are both phosphorylated commonly in somatic cells after irradiation (Figure S2). These results suggest that *Trp53* deficiency increases the survival of irradiated SSCs.

Comparison of the Radiation Response in Different Cell Types
To examine the differences in the damage responses of various cell types, we first used terminal deoxynucleotidyl
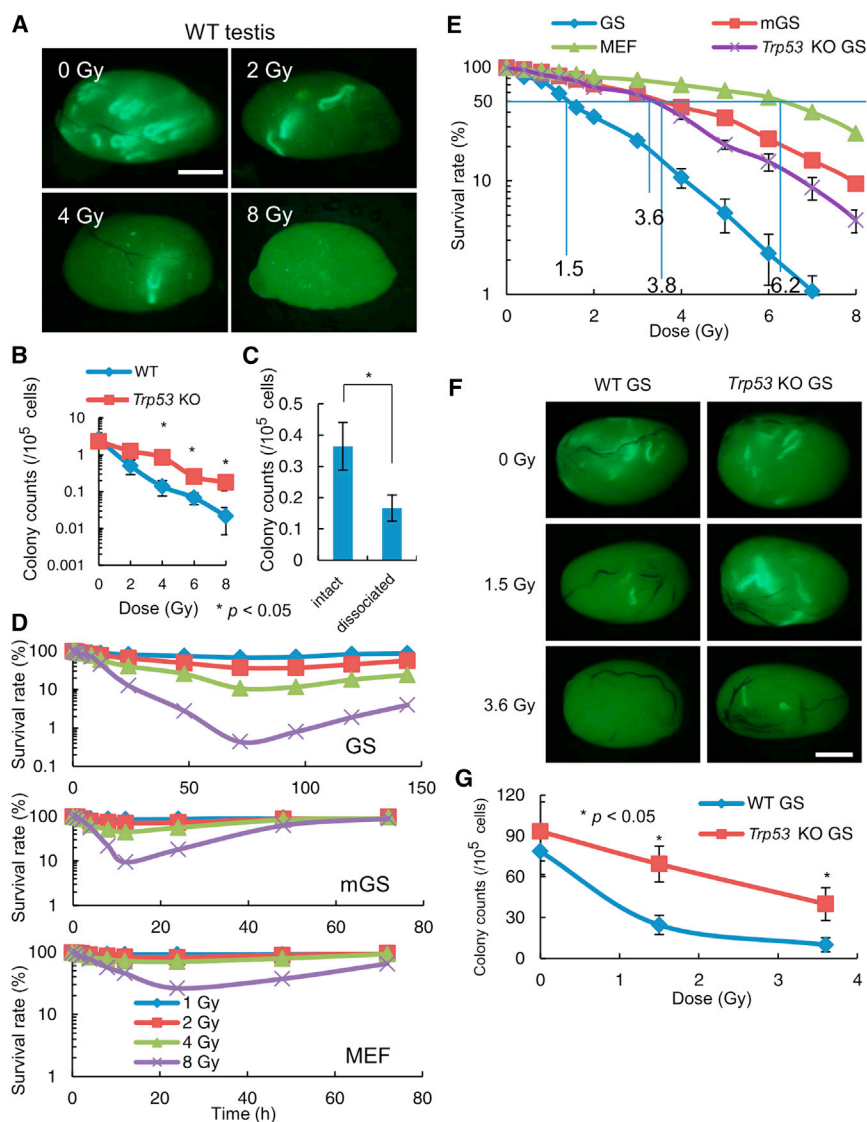
Figure 1. Radiation Response of SSCs and GSCs

(A) Macroscopic appearance of testes that received irradiated green mouse testes. Testis cells were dissociated into a single-cell suspension and irradiated at the indicated doses.

(B) Colony counts (n = 16–18 cells, 3 experiments).

(C) Increased survival of SSCs in intact testes. To prepare intact testis samples, green mouse testes were irradiated at 4 Gy and dissociated into single-cell suspensions (n = 24 testes, 3 experiments).

(D) Survival curves of GSCs, mGSCs, and MEFs. The cell survival rate was determined at the indicated time points after irradiation (n = 3 experiments). Cells were irradiated 1 day after plating.

(E) Radiation dose dependence of GSCs, mGSCs, and MEFs. The survival rate of GSCs, mGSCs, and MEFs was determined 72, 12, and 24 hr after irradiation, respectively, when the cells showed minimum cell recovery (n = 3 experiments). The LD_{50} of each cell type is indicated.

(F) Macroscopic appearance of testes that received WT and *Trp53* KO GSCs. WT and *Trp53* KO GSCs were irradiated at 1.5 and 3.6 Gy, respectively, and transplanted into recipient mouse testes.

(G) Colony counts (n = 13–15 testes; 3 experiments).

Data are represented as mean ± SEM. *p < 0.05. Scale bar, 1 mm (A and F). See also Figures S1 and S2.

transferase biotin-dUTP nick-end labeling (TUNEL) assays to quantify apoptosis (Figures 2A and 2B). The number of apoptotic mGSCs and MEFs increased transiently to ∼20%–25% within 4 hr and then declined to basal levels by 72 hr. In contrast, the number of apoptotic WT GSCs reached ∼50% at 12 hr after radiation and then declined gradually until ∼72 hr. Although *Trp53* KO GSCs also underwent apoptosis, the levels were significantly suppressed compared with those of WT cells. In addition, there was a different pattern of cell-cycle arrest (Figure 2C). Whereas MEFs showed a relative increase in G2/M phase after irradiation, the WT, but not *Trp53* KO, GSCs were arrested at G1. ESCs and mGSCs did not show apparent changes.

Next, cells were stained by γH2AX to detect DNA damage (Figure 2D). Both immunocytochemistry and western

blotting revealed that all cell types exhibited positive signals immediately after irradiation, but the number of γH2AX+ mGSCs was decreased significantly compared with other cell types. Moreover, γH2AX+ mGSCs and MEFs disappeared rapidly at 12–24 hr postirradiation (Figures 2E, 2F, and S2). In contrast, both WT and *Trp53* KO GSCs stained strongly, even at 72 hr postirradiation. Because ATM is an important mediator of the DNA damage response and also contributes to SSC self-renewal (Takubo et al., 2008), we examined its expression levels by western blotting (Figures S3A and S3B). Analysis revealed that GSCs express a low level of ATM compared with other cell types. Thus, DNA damage persists longer in GSCs than in mGSCs or MEFs, which may be the result of lower ATM levels.
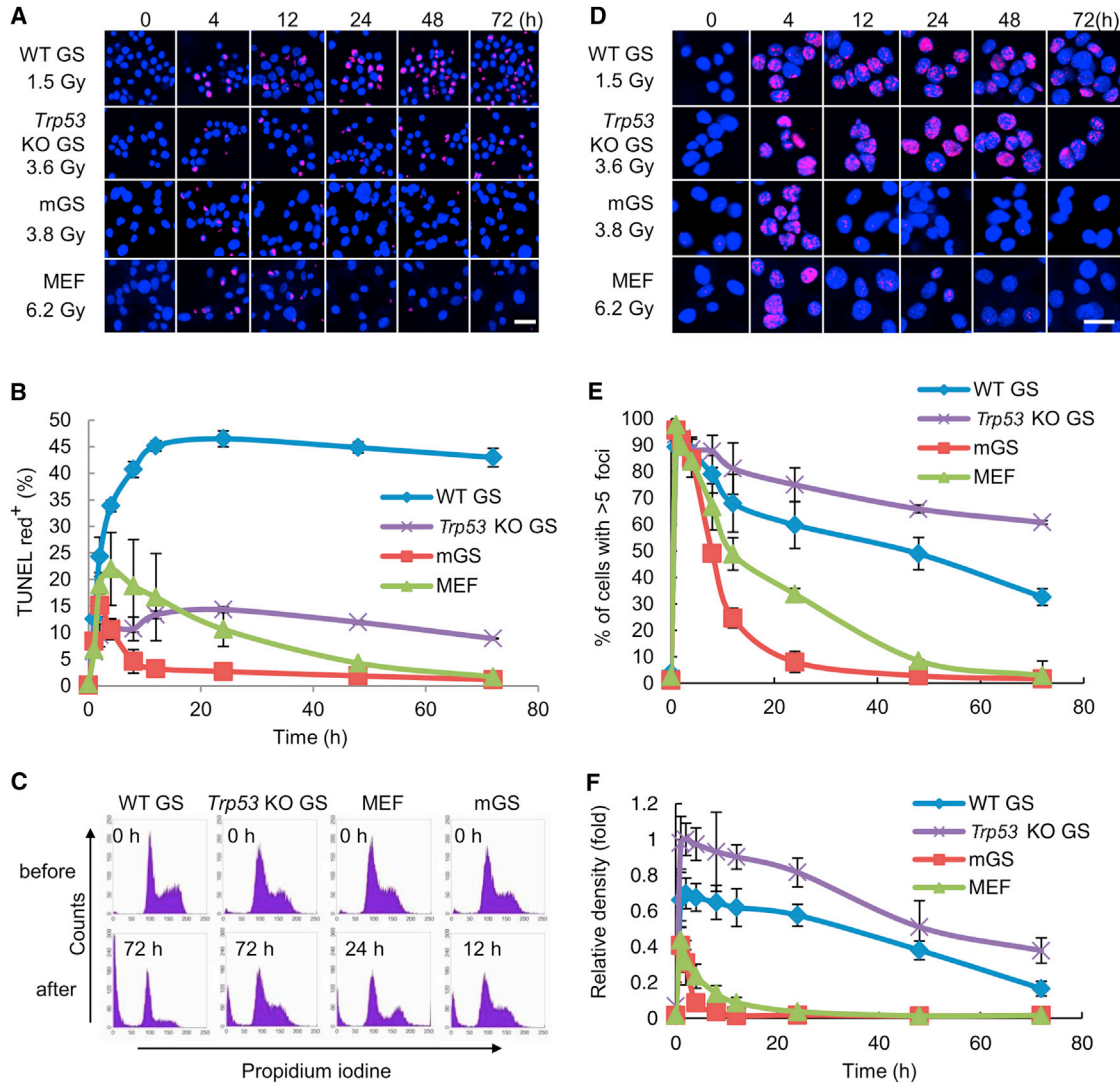
Figure 2. Damage Response of Different Cell Types after Irradiation

(A) TUNEL staining. Counterstained with Hoechst 33342.

(B) Quantification of apoptosis (n = 3 experiments). At least 1,500 cells were counted in each experiment.

(C) Analysis of cell cycle distribution. GSCs, mGSCs, and MEFs were recovered 72, 12, and 24 hr, respectively, after irradiation. WT GSCs were arrested at G1 phase, whereas MEFs were arrested at G2/M phase.

(D) γH2AX staining, counterstained with Hoechst 33342.

(E) Quantification of cells with six or more γH2AX foci (n = 3 experiments). At least 1,022 cells were counted at each time point.

(F) Quantification of western blot band intensities for γH2AX (n = 3 experiments).

Scale bar, 10 μm (A and D). Data are represented as mean ± SEM. See also Figures S2 and S3.

Increased GSC Survival after *Bbc3* Depletion

To understand the mechanism of action of *Trp53*, we examined several *Trp53* downstream target genes. *Trp53*-dependent cell-cycle control is determined largely by the *Trp53*-responsive *Cdkn1a*, which induces G1 and G2 growth arrest (Komarova et al., 2000). Because *Cdkn1a* is involved in radiation-induced apoptosis by interacting with proapoptotic molecules (Gartel and Tyner, 2002), we derived *Cdkn1a* KO GSCs and compared them with WT cells. However, there were no significant differences in survival after irradiation (Figure 3A).

Previous studies in somatic cells revealed that two different *Trp53*-regulated apoptotic pathways (intrinsic and extrinsic) protect against radiation (Haupt et al., 2003). *Bcl2* family members regulate the intrinsic pathway, and some of these are regulated transcriptionally by *Trp53*
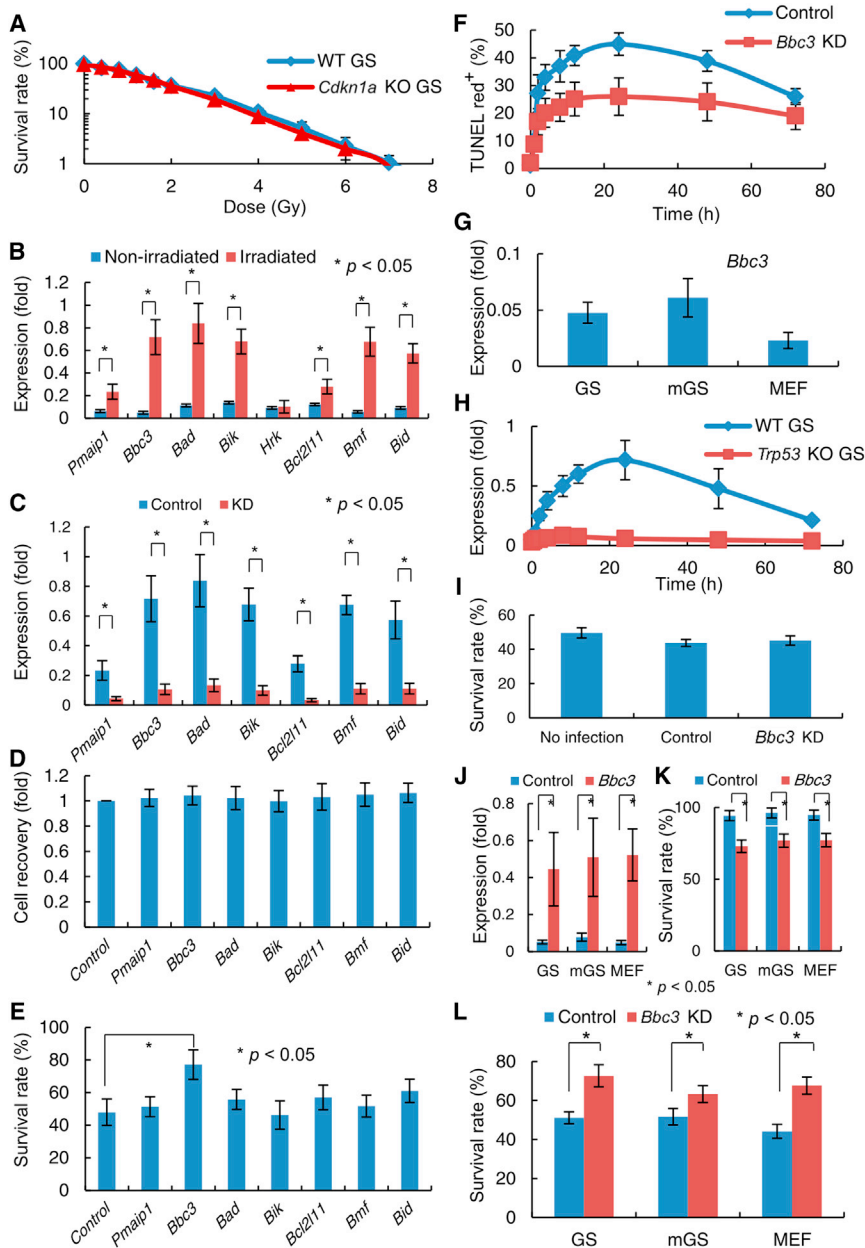
Figure 3. Involvement of *Bbc3* in GSC Survival after Irradiation

(A) Radiation sensitivity of *Cdkn1a* KO GSCs (n = 3 experiments).

(B) Real-time PCR analysis of BH3-only protein expression (n = 3 experiments). Cells were analyzed 24 hr after irradiation.

(C) Real-time PCR analysis of target gene expression following transfection of lentiviruses expressing shRNA (n = 3 experiments). Cells were irradiated 3 days after transfection and harvested the following day.

(D) Effects of target gene depletion on proliferation (n = 3 experiments). Cells were analyzed 3 days after transfection.

(E) Increased survival of irradiated GSCs after *Bbc3* depletion (n = 3 experiments). Cells were irradiated 3 days after transfection and assayed 3 days later.

(F) Quantification of apoptotic cells using TUNEL staining (n = 3 experiments). At least 1,500 cells were counted at each time point.

(G) Expression of *Bbc3* in several cell types (n = 3 experiments).

(H) Expression of *Bbc3* in irradiated WT and *Trp53* KO GSCs (n = 3 experiments).

(I) Survival of *Trp53* KO GSCs that were transfected with a *Bbc3* KD vector (n = 3 experiments). Cells were irradiated 3 days after transfection and cell survival was determined 3 days after irradiation.

(J) Real-time PCR analysis of *Bbc3* overexpression (n = 3 experiments). Cells were analyzed 3 days after transfection.

(K) Induction of apoptosis by *Bbc3* overexpression (n = 3 experiments). The survival rates of GSCs transfected as indicated are shown. Cells were assayed 3 days after transfection.

(L) Increased survival of different cell types after *Bbc3* depletion (n = 3 experiments). Cells were irradiated 3 days after transfection. GSCs, mGSCs, and MEFs were assayed 72, 12, and 24 hr after irradiation, respectively.

Data are represented as mean ± SEM. *p < 0.05. See also Table S1.

(Haupt et al., 2003). Because BH3-only proteins are implicated in radiation-induced apoptosis (Qiu et al., 2008; Yu et al., 2010), we investigated the expression of BH3-only proteins using real-time PCR 24 hr after irradiation (Figure 3B). GSCs showed increased expression of *Pmaip1*, *Bbc3*, *Bad*, *Bik*, *Bcl2l11*, *Bmf*, and *Bid*; however, *Hrk* did not change significantly.

We then used short hairpin RNA (shRNA) to assess the effect of gene depletion. Knockdown (KD) of any of these genes did not affect GSC proliferation (Figures 3C and

3D). However, *Bbc3* depletion increased GSC survival and decreased TUNEL staining (Figures 3E and 3F). The expression of *Bbc3* was comparable among cell types (Figure 3G). In GSCs, *Bbc3* was induced in a *Trp53*-dependent manner (Figure 3H), and *Bbc3* KD did not influence *Trp53* KO GSC survival (Figure 3I). Overexpression of *Bbc3* induced apoptosis in all cell types (Figures 3J and 3K). *Bbc3* KD also rescued apoptosis in mGSCs and MEFs (Figure 3L), suggesting that *Bbc3*-dependent apoptosis was not limited to germ cells.
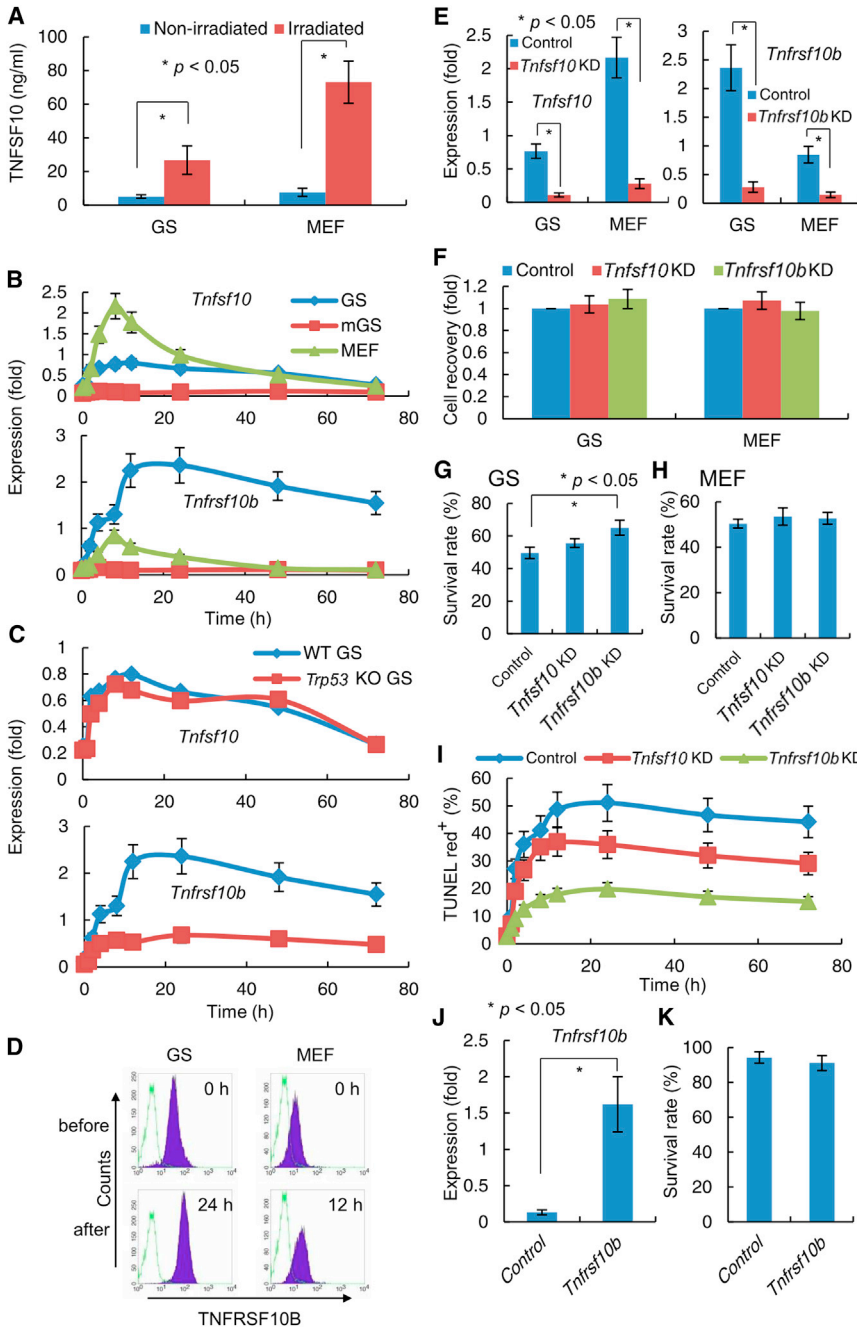
Figure 4. Involvement of TNFSF10-TNFRSF10B in GSC Survival after Irradiation

(A) *Tnfsf10* expression. Cells were plated 3 days before irradiation and protein levels were determined by ELISA (n = 3 experiments).

(B) Real-time PCR analysis of *Tnfsf10* and *Tnfrsf10b* expression after irradiation (n = 3 experiments).

(C) Real-time PCR analysis of *Tnfsf10* and *Tnfrsf10b* expression in WT and *Trp53* KO GSCs after irradiation (n = 3 experiments).

(D) Flow-cytometric analysis of TNFRSF10B expression after irradiation (purple population). Green lines indicate unstained controls.

(E) Real-time PCR analysis of target gene expression following transfection of lentiviruses expressing shRNA against *Tnfsf10* or *Tnfrsf10b* (n = 3 experiments). Cells were irradiated 3 days after transfection. GSCs and MEFs were assessed 24 and 8 hr after irradiation, respectively.

(F) Effect of *Tnfsf10* or *Tnfrsf10b* depletion on proliferation (n = 3 experiments). Cells were analyzed 3 days after transfection.

(G and H) Effects of *Tnfsf10* or *Tnfrsf10b* depletion on survival after irradiation on GSCs (G) and MEFs (H) (n = 3 experiments). Cells were irradiated 3 days after transfection, and GSCs and MEFs were recovered 72 and 24 hr after irradiation, respectively.

(I) Quantification of apoptosis (n = 3 experiments). At least 1,500 cells were counted at each time point.

(J) Real-time PCR analysis of *Tnfrsf10b* expression (n = 3 experiments). Cells were transfected with the indicated viral constructs and analyzed 3 days after transfection.

(K) Effect of *Tnfrsf10b* overexpression on GSC survival (n = 3 experiments). Cells were assayed 3 days after transfection with the indicated vectors.

Data are represented as mean ± SEM. *p < 0.05. See also Figure S4 and Table S1.

The TNFSF10-TNFRSF10B Pathway Is Involved in the Radiation Response of GSCs

The above data suggest the involvement of the intrinsic pathway in radiation-induced apoptosis. However, we noticed that the culture supernatant of irradiated GSCs and MEFs caused apoptosis in nonirradiated GSCs (Figure S4). Because the extrinsic pathway involves death receptors such as FAS, radiation-induced GSC apoptosis may also activate the extrinsic pathway. An ELISA showed that the expression of TNFSF10, a TNFRSF10B ligand, was increased significantly in the media of irradiated cells (Figure 4A). We then quantified the levels of *Tnfsf10* and *Tnfrsf10b* using real-time PCR (Figure 4B). *Tnfsf10* was induced more strongly in MEFs than in GSCs, whereas *Tnfrsf10b* was upregulated more strongly in GSCs than MEFs. The expression of *Tnfsf10* and *Tnfrsf10b* was unchanged in mGSCs. The induction of *Tnfrsf10b*, but not *Tnfsf10*, was *Trp53* dependent (Figure 4C). Flow-cytometric

analysis confirmed the upregulation of TNFRSF10B (Figure 4D).

We then depleted these genes using shRNA to assess their roles (Figure 4E). Depletion of either gene did not influence GSCs and MEF proliferation (Figure 4F). However, *Tnfrsf10b* depletion increased the survival of GSCs, but not MEFs (Figures 4G and 4H). Apoptosis was suppressed for at least 72 hr postirradiation (Figure 4I). Interestingly, *Tnfrsf10b* transfection did not induce apoptosis (Figures 4J and 4K). These results suggest that the TNFSF10-TNFRSF10B pathway contributes to radiation-induced GSC apoptosis.

Trp53-Independent Induction of *Tnfrsf10b* by *Trp53inp1*

Reactive oxygen species (ROS) generation accompanies irradiation treatment (Nagaria et al., 2013). Flow-cytometric analysis showed that radiation induced increased ROS levels in GSCs (Figure S5A). Addition of H_2O_2 also increased apoptosis of irradiated WT GSCs (Figure S5B). Because *Trp53inp1* is strongly expressed in the testis and is a major mediator of the antioxidant effects of *Trp53* (Cano et al., 2009), we hypothesized that *Trp53inp1* regulates the SSC radiation response. Real-time PCR analysis revealed that expression of *Trp53inp1* was higher in WT GSCs than in other cell types (Figure 5A) and was induced by irradiation (Figure 5B). This was *Trp53* dependent, because *Trp53inp1* induction was significantly reduced in *Trp53* KO GSCs (Figure 5C). In a manner similar to that observed for *Trp53*, *Trp53inp1* KD increased ROS levels when GSCs were supplemented with H_2O_2 (Figures S5C–S5E), which confirmed its antioxidant function (Cano et al., 2009).

Although *Trp53inp1* KD did not influence cell proliferation under nonirradiated conditions, it increased the survival of irradiated WT GSCs (Figures 5D–5F). *Trp53inp1* KD attenuated apoptosis for 72 hr postirradiation (Figure 5G). This protection was not observed in other cell types. Transfecting GSCs with *Trp53inp1* increased *Tnfrsf10b* expression in nonirradiated GSCs, whereas *Trp53inp1* KD downregulated *Tnfrsf10b* expression after irradiation (Figures 5H and 5I). Unlike the case with *Bbc3*, *Trp53inp1* overexpression did not induce apoptosis (Figures 5J and K).

In Vivo Expression of BBC3, TNFRSF10B, and TRP53INP1 in Undifferentiated Spermatogonia after Irradiation

To confirm the in vitro data, we examined the expression of BBC3, TNFRSF10B, and TRP53INP1 using antibodies against GFRA1 and CDH1 (Figure 6A). GFRA1 was highly expressed in A_s and A_{paired} spermatogonia and gradually declined in $A_{aligned}$ spermatogonia. CDH1 was expressed

in the total undifferentiated spermatogonia population (Tokuda et al., 2007). BBC3 and TNFRSF10B were expressed in ~2%–4% of $CDH1^+$ or $GFRA1^+$ cells in nonirradiated testes. TRP53INP1 was expressed more widely in ~19% and ~11% of $CDH1^+$ and $GFRA1^+$ cells, respectively (Figure 6B). Unlike other proteins, TNFRSF10B showed focal expression, which is probably due to accumulation in lipid rafts (Min et al., 2009).

All of these markers were strongly expressed 24 hr after irradiation (Figure S6A), but TNFRSF10B and TRP53INP1 were upregulated more significantly than BBC3 in both $CDH1^+$ and $GFRA1^+$ cells (Figure 6B). Although the expression of BBC3 in germ cells was increased significantly (Figure S6A), its expression in undifferentiated spermatogonia remained at ~3% (Figure 6B). Double immunohistochemistry showed that BBC3 was upregulated significantly in KIT^+ spermatogonia (Figures S7A and S7B). In addition, western blotting showed that TNFSF10 increased in irradiated testes from WBB6F1-W/W^v (W) mice, which lack differentiating germ cells (Figures S6B and S6C).

Functional Analysis of SSC Activity Using Germ Cell Transplantation

We performed germ cell transplantation to confirm the effects of *Bbc3*, *Tnfrsf10b*, and *Trp53inp1* on SSCs. First, green GSCs were transfected with a KD vector for each gene and irradiated 3 days after transfection. Cells were then microinjected into the seminiferous tubules (Figure 7A). Analysis of recipient testes showed that the concentration of SSCs ranged from 0.1% to 0.7%, which is somewhat lower than previously reported values for GSCs (1%–2%) (Kanatsu-Shinohara et al., 2005). The lower colonization efficiency is likely due to the transfection treatments we used in this study. Surprisingly, *Bbc3*-depleted cells generated fewer colonies than control cells, suggesting that *Bbc3* is not involved in radiation-induced SSC apoptosis. In contrast, *Tnfrsf10b* or *Trp53inp1* KD improved GSC survival (Figure 7A) and normal spermatogenesis was observed in transplanted cells (Figure 7B). Because progenitor cells do not have self-renewal activity and "disappear" after transplantation, only SSCs can produce this result. Therefore, the GSCs that survived after *Bbc3* KD were enriched for progenitor cells.

Next, we confirmed the effect of *Tnfrsf10b* and *Trp53inp1* using KO mice. KO mice were mated with green mice to introduce a donor marker. The mice were irradiated at 6 Gy and their testes were used for transplantation. Analyses of the recipient testes showed that deficiency of *Tnfrsf10b* or *Trp53inp1* increased donor cell survival significantly after irradiation (Figures 7C and 7D). Immuno-histochemistry revealed that apoptosis was reduced in
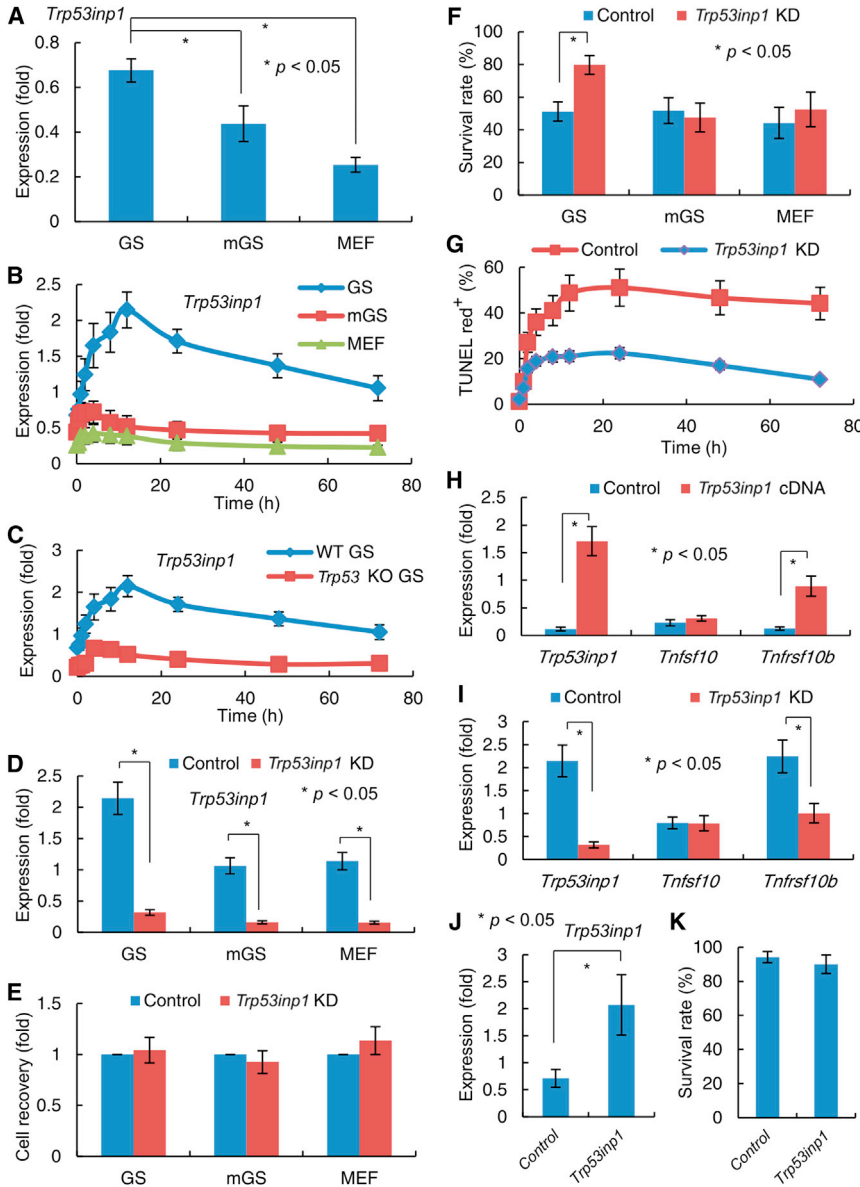
Figure 5. Involvement of *Trp53inp1* in GSC Survival after Irradiation

(A) Real-time PCR analysis of *Trp53inp1* expression in different cell types (n = 3 experiments).

(B) Real-time PCR analysis of *Trp53inp1* expression after irradiation (n = 3 experiments).

(C) Real-time PCR analysis of *Trp53inp1* expression in WT and *Trp53* KO GSCs after irradiation (n = 3 experiments).

(D) Real-time PCR analysis of *Trp53inp1* expression following transfection of lentivirus expressing shRNA against *Trp53inp1* (n = 3 experiments). Cells were transfected with a *Trp53inp1* KD vector and irradiated 3 days after transfection. Cells were analyzed 12 hr after irradiation.

(E) Effect of *Trp53inp1* depletion on proliferation (n = 3 experiments). Cells were assessed 3 days after transfection.

(F) Effect of *Trp53inp1* depletion on survival after irradiation (n = 3 experiments). Cells were irradiated 3 days after transfection. GSCs, mGSCs, and MEFs were analyzed 72, 12, and 24 hr after irradiation, respectively.

(G) Quantification of apoptosis (n = 3 experiments). At least 1,500 cells were counted at each time point.

(H) Real-time PCR analysis of *Trp53inp1*, *Tnfsf10*, and *Tnfrsf10b* expression following *Trp53inp1* overexpression (n = 3 experiments). Cells were assessed 3 days after transfection.

(I) Real-time PCR analysis of *Trp53inp1*, *Tnfsf10*, and *Tnfrsf10b* expression following *Trp53inp1* depletion and irradiation (n = 3 experiments). Cells were irradiated 3 days after transfection and analyzed 3 days after irradiation.

(J) Real-time PCR analysis of *Trp53inp1* overexpression (n = 3 experiments). Cells were transfected with the indicated viral constructs and assayed 3 days after transfection.

(K) Effects of *Trp53inp1* overexpression on GSC survival (n = 3 experiments). Cells were analyzed 3 days after transfection of indicated vectors.

Data are represented as mean ± SEM. *p < 0.05. See also Figure S5 and Table S1.

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both CDH1^{+} and GFRA1^{+} undifferentiated spermatogonia in irradiated *Tnfrsf10b* and *Trp53inp1* KO mouse testes (Figures 7E and 7F). TNFRSF10B and TRP53INP1 were not detected in irradiated *Trp53* KO testes, confirming the results from GSCs (Figure 7G). Only 0.4%–0.8% of CDH1^{+} or GFRA1^{+} cells expressed TNFRSF10B or TRP53INP1 in *Trp53* KO mice (Figure 7H). In contrast, TNFRSF10B and TRP53INP1 were expressed in >88.6% of CDH1^{+} or GFRA1^{+} cells in WT mice. These results suggest that the

Trp53-Trp53inp1-Tnfrsf10b pathway is responsible for radiation-induced SSC apoptosis in vivo.

Rescuing GSCs from Anticancer-Reagent-Induced Cell Death by *Trp53inp1* or *Tnfrsf10b* Depletion

The rescue of radiation-induced apoptosis by *Trp53inp1* or *Tnfrsf10b* depletion suggested that inhibition of these genes could prevent damage induced by other genotoxic insults. Therefore, we examined the effect of these genes
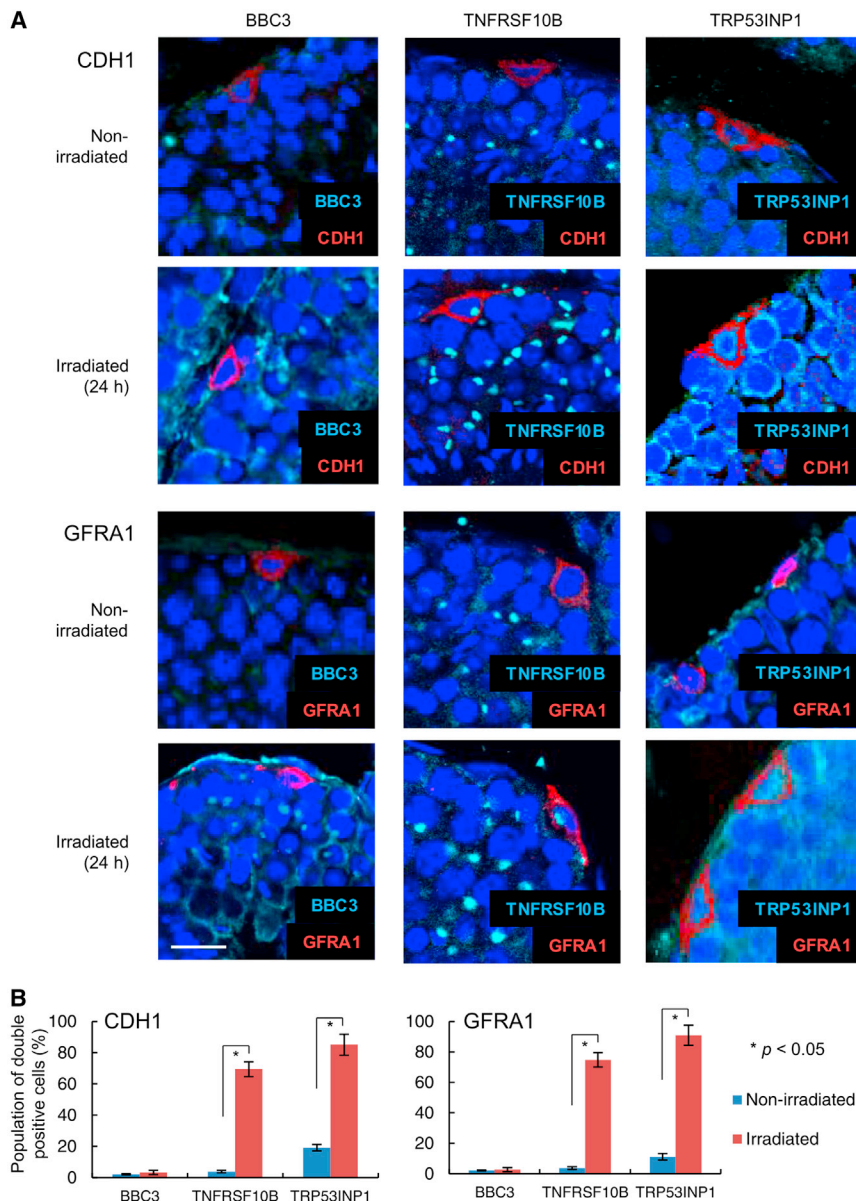
Figure 6. Induction of *Bbc3*, *Tnfrsf10b*, and *Trp53inp1* in Irradiated WT Testes

(A) Immunohistochemical staining of BBC3, TNFRSF10B, and TRP53INP1 in WT testes 24 hr after irradiation.

(B) Quantification of cells using undifferentiated spermatogonia marker expression (n = 3 experiments). At least 150 cells expressing each spermatogonia marker were counted.

Data are represented as mean ± SEM. *p < 0.05. Scale bar, 10 μm (A). See also Figures S6 and S7.

on damage induced by cisplatin or mitomycin C. Both of these reagents crosslink DNA and are used as anticancer agents, and cisplatin is commonly used to treat germ cell tumors (Wu et al., 2004). GSCs were transfected with shRNA against *Trp53inp1* or *Tnfrsf10b*, and cisplatin or mitomycin C was added 3 days later. The cells were analyzed 72 hr later after addition of chemicals. GSCs treated with 80 μg/ml mitomycin C exhibited significantly enhanced survival after *Tnfrsf10b* or *Trp53inp1* depletion. Cell survival after cisplatin treatment was improved at all tested doses, and the differences were statistically significant with >2 μg/ml (Figures 7I), suggesting that inhibiting the *Trp53inp1-Tnfrsf10b*

pathway assists in protecting SSCs from chemical-induced damages.

DISCUSSION

In contrast to previous studies, we have shown increased survival of *Trp53* KO SSCs after irradiation. At least two factors must be considered to explain this discrepancy. First is the method used to identify SSCs. Whereas we identified SSCs using a functional transplantation assay, previous studies identified them by observing in situ colony regeneration. It is challenging to distinguish whether poor colony

development is due to defects in the SSCs or their microenvironment. Second is the abnormal cell division of *Trp53* KO spermatogonia. A previous study showed that loss of *Trp53* caused a 40%–50% increase in the total number of type A spermatogonia in nonirradiated testes and also stimulated a 7-fold increase in the number of giant spermatogonia after irradiation (Beumer et al., 1998). Giant spermatogonia were mostly single cells, suggesting their A_s spermatogonia origin. The fate of giant spermatogonia is unknown, but it is possible that they represent SSCs that cannot differentiate normally. Because TRP53 expression is highest in meiotic cells (Hendry et al., 1996), it is also possible that *Trp53* KO cells experience difficulties in undergoing meiotic differentiation. Although we did not analyze the effects of *Trp53* signaling on spermatocyte or spermatid development, this may result in smaller colony formation, which could be underrepresented by a histological detection method.

To analyze the molecules downstream of *Trp53*, we first focused on the role of CDKN1A and the BH3-only protein family. Several previous studies suggested that CDKN1A-mediated cell-cycle arrest and *Bbc3*-induced apoptosis contribute to radiation damage in self-renewing tissues (Yu et al., 2003; Leibowitz et al., 2011). For example, DNA damage in hematopoietic and mammary stem cells activated CDKN1A and induced symmetrical self-renewal divisions by suppressing *Trp53* activation, thus inhibiting apoptosis (Insinga et al., 2013). Moreover, *Bbc3* deficiency protected both intestinal crypt cells and hematopoietic stem cells from radiation damage (Yu et al., 2010; Qiu et al., 2008). In our study, *Cdkn1a* KO GSCs were comparable to WT GSCs, whereas *Bbc3* depletion enhanced GSC survival. Cell rescue by *Bbc3* depletion could also be applicable to MEFs and mGSCs, suggesting that this effect of *Bbc3* is not specific to germ cells.

Interestingly, our transplantation study revealed that *Bbc3* depletion could not protect SSCs, suggesting that the cells that survived irradiation were spermatogonia progenitors. *Bbc3*-depleted cells retained their proliferative activity but lost their recolonizing ability. This result was unexpected because several studies showed the involvement of *Bbc3* in the *Trp53*-induced depletion of adult stem cells, including spermatogonia (Qiu et al., 2008; Liu et al., 2010; Yu et al., 2010). An additional study reported that *Bbc3* is upregulated in ITGA6+ side population (SP) phenotype spermatogonia, which are thought to be SSC enriched (Coureuil et al., 2010). The viability of *Bbc3* KO SP cells was decreased by 1.6-fold, compared with a 2.7-fold reduction in the WT SP cells. However, because the presence of SSCs was not assessed in that study, it was unclear whether apoptosis occurred in SSC or progenitor cells. Our results suggest that *Bbc3* is upregulated in KIT+ cells and that radiation-induced apoptosis of progenitor cells,

rather than SSCs, may be *Bbc3* dependent. Given the beneficial roles of *Cdkn1a* and *Bbc3* in other self-renewing tissues, SSCs appear to have radiation-response features distinct from these stem cell types despite their common dependence on *Trp53*.

Another possible regulator of *Trp53*-mediated apoptosis is the TNFSF10-TNFRSF10B pathway. In stressed conditions, germ cell apoptosis depends on both extrinsic and intrinsic pathways. Radiation-induced apoptosis caused by DNA damage generally requires the intrinsic, rather than the extrinsic, pathway (Forand and Bernardino-Sgherri, 2009). One study showed that spermatogonia upregulated *Tnfrsf10b* after irradiation, whereas *Tnfsf10* deficiency could not inhibit radiation-induced apoptosis as efficiently as *Bbc3* deficiency (Coureuil et al., 2010). Consistent with this, the TNFSF10-TNFRSF10B pathway did not protect against apoptosis in gonocytes, which are spermatogonia precursors (Forand and Bernardino-Sgherri, 2009). Nevertheless, radiation-induced apoptosis in GSCs was rescued by depleting *Tnfrsf10b*, highlighting the importance of the extrinsic pathway in SSC radioprotection. Because TNFSF10 expression is upregulated in both germ cells and somatic cells, we speculate that increased TNFRSF10B expression in SSCs makes them more sensitive to apoptosis induced by upregulated TNFSF10 expression. Taken together, our results suggest that quality control of the male germline against genotoxic damage is unique in that differentiation induces a switch in the cell death machinery.

Although the induction of *Tnfrsf10b* is *Trp53* dependent, up to now, its mechanism of induction has been elusive. Our study reveals that *Trp53inp1* induces *Tnfrsf10b*. Despite its ubiquitous expression, suppression of *Trp53inp1* could rescue GSCs, but not mGSCs or MEFs, suggesting that it may confer a germ cell-specific response. *Trp53inp1* is induced by ROS (Cano et al., 2009) and was previously isolated as a *Trp53*-inducible protein that participates in *Trp53*-dependent apoptosis by regulating *Trp53* function (Okamura et al., 2001). More recent studies showed that it also regulates ROS and autophagy (Cano et al., 2009; Sancho et al., 2012; Seillier et al., 2012). TRP53INP1 binds to HIPK2, PRKCD, and TRP53 to mediate the phosphorylation of TRP53 at Ser46. Colocalization of these proteins in promyelocytic leukemia nuclear bodies facilitates the protein interactions. This increases TRP53 stability and transcriptional activity, leading to transcriptional activation of *Trp53* target genes such as TP53AIP1, cell growth arrest, and apoptosis upon DNA damage stress (Tomasini et al., 2003; Yoshida et al., 2006).

Trp53inp1 is a major mediator of the antioxidant function of *Trp53* (Cano et al., 2009). Although *Trp53inp1* KO mice do not exhibit an overt phenotype, they are susceptible to induction of colorectal tumorigenesis and acute
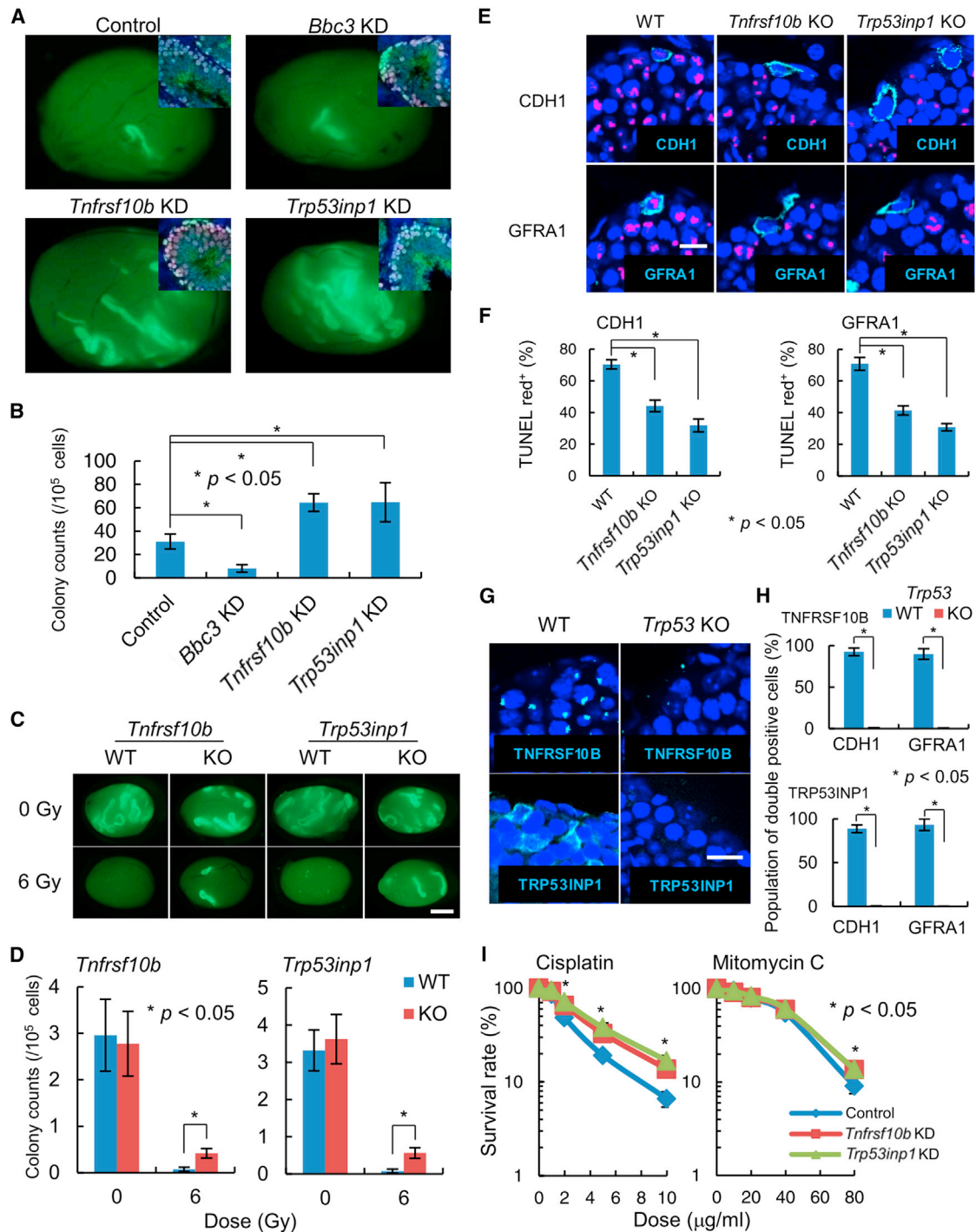
Figure 7. Functional Analysis of SSC Activity by Germ Cell Transplantation

(A) Macroscopic appearance of testes that received irradiated GSCs transfected with the indicated KD vectors. WT GSCs were irradiated at 1.5 Gy and transplanted into recipient mouse testes. Inset: immunohistochemical staining of SYCP3 (red) in recipient testes, counterstained with Hoechst 33342.

(B) Colony counts (n = 18 testes, 3 experiments).

(C) Macroscopic appearance of testes that received irradiated *Tnfrsf10b* or *Trp53inp1* KO testis cells. Donor testis cells were irradiated with 6 Gy and transplanted into recipient mouse testes.

(D) Colony counts (n = 10–18 testes, 3 experiments).

(legend continued on next page)

colitis, which is thought to be due to increased ROS production (Gommeaux et al., 2007). In a previous study (Morimoto et al., 2013), we showed that although moderate ROS levels were required to stimulate self-renewal, increasing ROS levels by H_2O_2 supplementation did not increase SSC activity, and high concentrations of H_2O_2 killed the SSCs. In this context, the degree of ROS generation after irradiation was apparently toxic. Our results suggest that *Trp53inp1* also induces *Tnfrsf10b* expression after irradiation. Because *Tnfrsf10b* is expressed more strongly in GSCs, such cells are probably more sensitive to apoptosis stimuli, which may explain the germ cell-specific rescue of radiation-induced apoptosis. In addition, the relatively lower ATM levels in GSCs also may have contributed to this phenomenon. Like *Trp53* KO mice, *Trp53inp1* KO mice have no apparent fertility phenotype. However, given *Trp53inp1*'s diverse functions and functional redundancy with *Trp53inp2* (Nowak et al., 2009), future studies are needed to delineate the physiological roles of these genes in germ cell biology.

It has been known for years that germ cells are highly sensitive to genotoxic reagents. This high level of apoptosis may provide an important mechanism to prevent abnormal germ cells from transmitting genetic information to the next generation. In this context, our results suggest that *Trp53* appears to contribute to quality control. Although the involvement of *Trp53* in the radiation response of SSCs was not confirmed in previous studies, our results suggest that it is a critical molecule in the genotoxic response of SSCs. However, several issues remain to be addressed. For example, although the *Trp53-Bbc3* pathway has been shown to be involved in protection of somatic stem cells, it is not known whether the *Trp53-Trp53inp1-Tnfrsf10b* apoptotic pathway plays any role in different self-renewing tissues. It will also be valuable to determine the physiological role of *Trp53inp1* during spermatogenesis. TRP53INP1 was expressed in both CDH1^{+} and GFRA1^{+} spermatogonia without irradiation. Given the increased apoptosis of spermatogenic cells in *Trp53* KO mice, TRP53INP1 may be used to eliminate abnormal germ cells during spermatogenesis. Thus, our analyses based on SSC culture and transplantation techniques provide the groundwork for studying the DNA damage response in SSCs, and suggest that distinct cell death machineries are activated during differentiation in spermatogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture

GSCs were established from green mice (a gift from Dr. M. Okabe, Osaka University) or from *Cdkn1a* KO mice (The Jackson Laboratory), both of which were bred into an ICR background for at least seven generations. GSCs from ICR background *Trp53* KO mice were previously described (Kanatsu-Shinohara et al., 2004). GSCs were also derived from 7-day-old WT ICR and DBA/2 mice (Japan SLC). The GSCs were maintained as described previously (Kanatsu-Shinohara et al., 2003). The mGSCs used in this study were described previously (Kanatsu-Shinohara et al., 2004). ESCs (R1 cell line, a gift from Dr. M. Ikawa, Osaka University) and mGSCs were cultured in standard ESC culture medium, which consisted of Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 15% fetal bovine serum (FBS) and 10^3 U/ml leukemia inhibitory factor (ESGRO; Invitrogen). MEFs were prepared from 12.5–14.5 days postcoitum ICR mouse embryos and cultured in DMEM with 10% FBS. GSCs were maintained on laminin (20 µg/ml; BD Biosciences)-coated dishes at a density of $3.0 \times 10^5/9.6$ cm^2, whereas ESCs, mGSCs, and MEFs were maintained on gelatin-coated plates at a density of $1.0 \times 10^5/9.6$ cm^2. Cell viability was determined by adding 0.4% trypan blue (Invitrogen). To collect culture supernatant, GSCs or MEFs were cultured in StemPro-34 SFM supplemented with 1% FBS for 3 days before irradiation. Culture supernatant was collected from MEFs and GSCs 1 and 3 days after radiation, respectively. The culture supernatant was supplemented with 15 ng/ml GDNF and 10 ng/ml FGF2 (both from Peprotech) before use. Mitomycin C (Sigma) and cisplatin (Wako) were reconstituted in PBS and used at the indicated doses.

Statistical Analysis

Results are presented as means ± SEM. Significant differences between means for single comparisons were identified using Student's t test. Multiple-comparison analyses were performed using ANOVA followed by Tukey's HSD test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with

(E) Immunohistochemistry for CDH1 or GFRA1 with TUNEL in irradiated *Tnfrsf10b* or *Trp53inp1* KO mouse testes. *Tnfrsf10b* and *Trp53inp1* KO mice were irradiated with 2 Gy, and then used for immunohistochemistry 24 hr after irradiation.
(F) Number of CDH1^{+} or GFRA1^{+} cells undergoing apoptosis (n = 3 experiments). At least 96 cells in 32 tubules were counted.
(G) Double immunohistochemistry of CDH1 or GFRA1 with TNFRSF10B or TRP53INP1 in irradiated *Trp53* KO mouse testes. *Trp53* KO mice were irradiated with 2 Gy and used for immunohistochemistry 24 hr after irradiation.
(H) Quantification of cells with undifferentiated spermatogonia marker expression and TNFRSF10B or TRP53INP1 expression (n = 3 experiments). At least 106 cells expressing each spermatogonia marker were counted in each experiment.
(I) Survival of GSCs after mitomycin C or cisplatin treatment (n = 3 experiments).
Data are represented as mean ± SEM. *p < 0.05. Scale bars, 1 mm (A and C) and 10 µm (E and G).

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